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Award Number: W81XWH-09-1-0661

TITLE: Intraoperative Molecular Imaging for Rapid Assessment of Tumor Margins

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REPORT DATE: September 2011

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-09-2011	Annual Summary	1 SEP 2010 - 31 AUG 2011

4. TITLE AND SUBTITLE
Intraoperative Molecular Imaging for Rapid Assessment of Tumor Margins

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0661

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Dartmouth College
Hanover, NH 03755

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The objective of this research is to develop methods for rapid detection of tumor margins during breast cancer surgery using targeted fluorescence-labeled tracers and thus reduce the high rate of repeat surgery. We have developed and characterized a spectrometer-based fluorescence probe system to quantify fluorescence signals in excised tissue. Spectral processing techniques allowed the removal of contaminating tissue auto-fluorescence and provided highly linear responses to reporter concentration. Additionally, a new dual-reporter compartmental model was used to demonstrate quantification of receptor-to-imaging agent binding potential in tumor models. This approach can be used in fluorescence guided surgery to dramatically enhance the tumorto-background contrast by suppressing the influence of fluorescence reporter kinetics and can reveal the availability of receptor targets for therapy.

15. SUBJECT TERMS
fluorescence, molecular imaging, surgery, breast cancer, fluorescence guided surgery, animal models

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	12	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction

The scientific objective of this research is to explore the feasibility of assessing tumor margin status during breast cancer surgery using targeted fluorescence-labeled tracers and thus reduce the rates of repeat surgery. We hypothesize that targeted fluorescent drugs coupled with an appropriate, rapid fluorescence imaging system will inform the surgeon or surgical pathologist of tumor margin status in excised specimens during the surgical procedure. The primary aims involve investigating topical application of a targeted fluorescence reporter to the excised tissue specimen to identify involved tumor margins. We are also studying the behavior of targeted reporters *in vivo* as an alternative to the topical application approach.

Body

Progress has been made toward the proposed aims of this project including the development of a fluorescence probe system for quantifying fluorophore concentration on *in vivo* or excised tissue specimens (Tasks 1 and 3). Given the importance of specific targeted binding for imaging tumor margins, we have also developed a new approach to determine receptor-to-imaging agent binding potential *in vivo* and demonstrated the approach in animal models using a the MRI-FMT imaging system (Task 5). A description of the primary accomplishments and ongoing efforts follows.

Spectrometer-based probe system for quantifying fluorescence activity in tissue

One of the challenges in imaging fluorescence in tissue is the often dominant contamination caused by tissue autofluorescence and non-specific binding. In many cases, spectral techniques can account for these confounding factors. To that end, we have developed a spectrally-resolved probe-based system for localized quantification of fluorophore concentration on living or excised tissues to help validate surgical broad-beam imaging configurations. The system, depicted in Fig. 1, consists of a bifurcated fiber probe with one branch attached to a laser source and the other to a cooled spectrometer (Ocean Optics QE65000) through a filtering unit. In the initial development stages, a 405 nm laser was used as the excitation source. Excitation filters were chosen to allow a portion of the laser light to pass through to the detector and serve as a reference in the spectrum. Using a 405 nm laser, the laser line was visible as a second order peak at 810 nm, as shown in Fig. 2(a).

Tissue autofluorescence contaminates the measured signal and reduces the sensitivity of the measurements at low fluorophore concentrations. To account for this, we have incorporated spectral fitting routines which use pre-measured spectral shapes to separate the contribution of the fluorophore and tissue autofluorescence. We have found this improves sensitivity by factor 10 – 100. Additionally, computing ratios of data from different parts of the same spectrum, such as the fluoresce-to-excitation ratio, dramatically reduces variability due to light coupling, laser intensity, detector sensitivity and light propagation through tissue. We are currently using phantom data to test several different ratios, including fluorescence-to-excitation and fluorescence-to-autofluorescence intensity, to determine the most robust measurement.

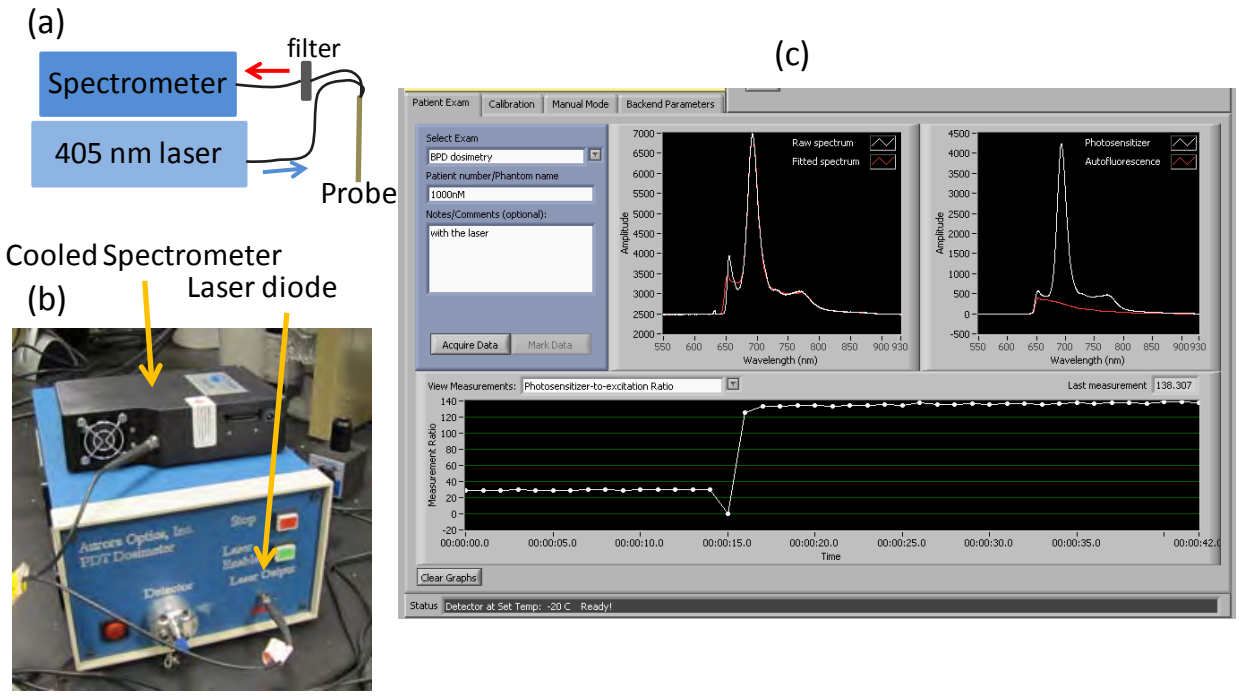
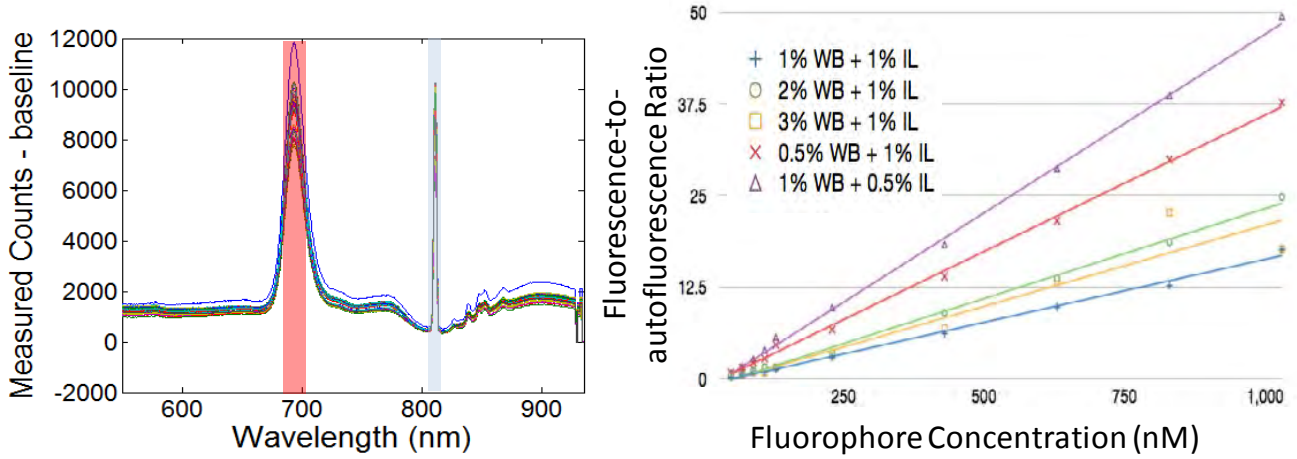


Figure 1. A diagram of the spectrometer-based fluorescence probe system is shown in (a) and a photograph in (b). A screenshot of the front panel control software showing the raw and processed spectra as well as a calibrated ratio measurement.

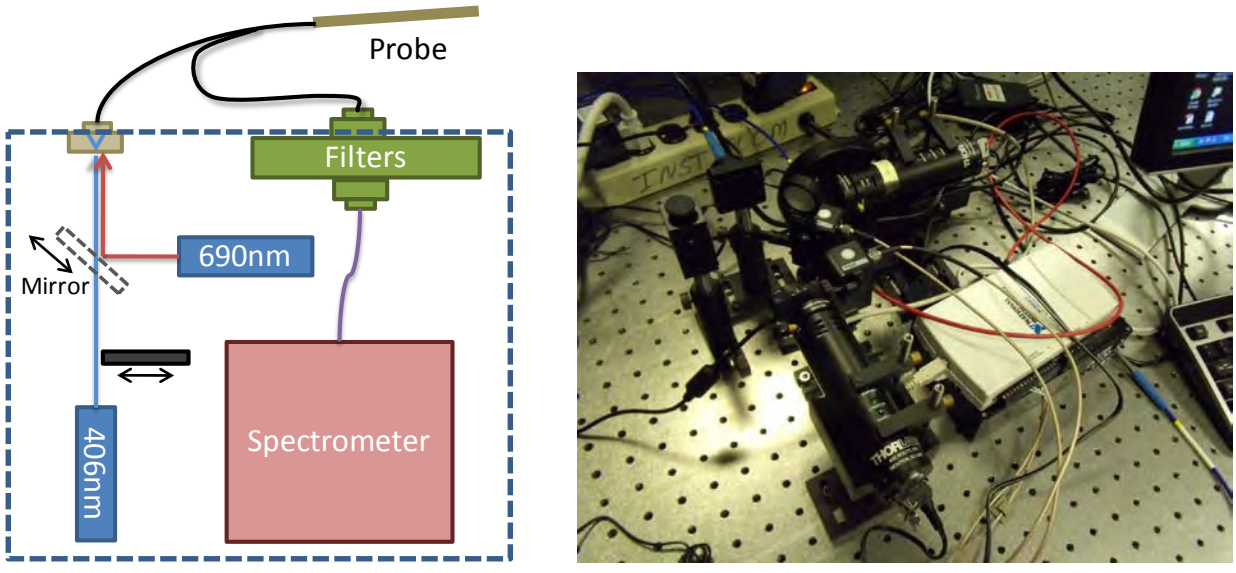
A critical requirement for quantifying drug concentration in tissue is that the measurements are robust and repeatable for tissues with different optical properties. We explored the stability of the measurement system in liquid phantoms containing different concentrations of whole blood and intralipid (to simulate changes in tissue scattering) and the fluorophore benzoporphyrin derivative (BPD). Figure 2 (b) shows the system response to changing fluorophore concentrations for different concentrations of whole blood and scattering. While the linearity of the response was excellent for a given phantom solution, the variance in the slopes between different solutions suggests that the ratio measurements were not adequately accounting for changes in optical properties. Thus, we determined that more sophisticated measurement tools were needed to produce a more robust system.



(a) (b)

Figure 2. (a) Example spectra showing the fluorescence peak (red bar) and the second order excitation peak for the 405 nm laser (in blue). The fluorescence-to-excitation or fluorescence-to-autofluorescence ratio were calculated as a surrogate measure of fluorophore concentration. This ratio is plotted as a function of fluorophore concentration in (b) for different concentrations of blood and intralipid in the solution, showing the impact that blood has on the ratio.

To mitigate the effects of optical properties, we are currently developing a second generation probe system with an additional light source channel and an automated filter set, shown in Fig. 3. Using an excitation source wavelength closer to the emission wavelength should ensure that the optical properties of both experienced by the excitation and emission fields are similar and thus changes in optical properties should have less of an effect on the measured ratio. This system is near completion and will be tested with the next two weeks. Should this not adequately account for the tissue optical properties, the additional channel may be used to incorporate a white light source which can be used to measure the tissue optical properties explicitly.

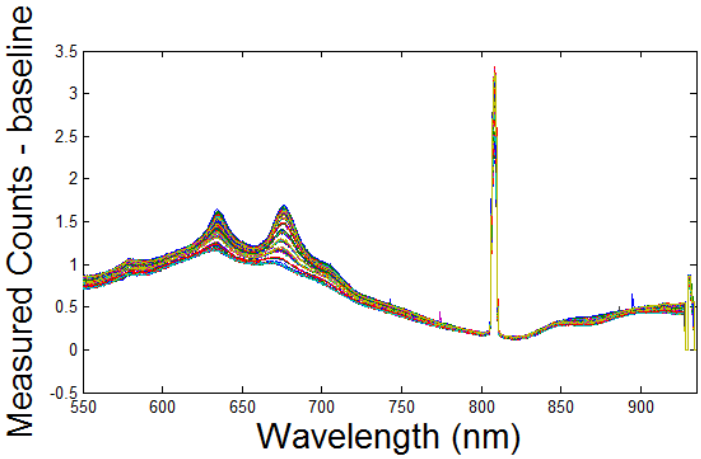


(a) (b)

Figure 3. Schematic (a) and photograph (b) of the next generation fluorescence probe system designed to

quantify fluorophore concentration on excised tissue.

The probe system was used to measure fluorescence spectra from excised breast tumors. A small population of mice was implanted with MTGB tumors in the mammary fat pad. Once the tumors grew to 0.7 cm across, the animal was sacrifice and the tumors immediately removed and measured with the probe system. Once a baseline measurement was acquired, a high concentration of aminoluvelinic acid (ALA), a compound which often causes enhanced production of the endogoneous fluorescent compound protoporphyrin IX in tissue, was applied topically to the tumor. The probe was then used to record the fluorescence spectra over 30 minutes following ALA application. Endogenous PPIX was present in measureable quantities even before the application of ALA, as shown in Fig. 4 (a), and the application of ALA did not appreciably change the fluorescence activity of the tissue. In addition to investigating non-ALA induced PPIX fluorescence as a tumor marker, we are currently using this approach to investigate other topically applied fluorophores in tumor tissue.



(a)

Animal	Pre-ALA	Post-ALA
1	3.23	2.93
2	2.34	2.65
3	3.72	3.61
4	2.28	2.39

(b)

Figure 4. The fluorescence spectrum of a breast tumor specimen measured immediately after resection using the fluorescence probe system is shown in (a). A portion of the fluorescence between 630 and 720 nm is due to the presence of the endogenous compound PPIX. In (b), probe measurements of excised breast tumors before and after topical application of ALA show little change from the presence of ALA.

Quantifying receptor binding potential using dual-reporter imaging

The ability to quantify the binding potential (BP) of a receptor-targeted imaging agent *in vivo* would have broad implications for surgical guidance and pre-clinical research of target-specific imaging. In this study, the binding potential is considered the product of the binding affinity (between the receptor and targeted agent) and the receptor density. Most optical imaging studies using receptor-targeted reporters in tissue use fluorescence intensity as a surrogate measure of receptor-agent binding. However, this simple approach does not account for the kinetics of the reporter in the tissue which is heavily influenced by the plasma clearance rate, the transport of the reporter into and out of the tissue from the bloodstream, and the rate of binding in the tissue. Given these confounding factors, fluorescence intensity of a single tracer alone does not provide the necessary information to quantify BP.

Recent work in our lab has demonstrated the ability to accurately quantify BP in vivo using a reference tracer model describing the kinetics of two fluorescent tracers administered simultaneously, one targeted and the other non-targeted. This compartmental model system is based on the simplified reference

tissue model introduced to the positron emission tomography (PET) community by Lammerstma(*1*), and uses comparisons between radionuclide activity in two tissue types to quantify binding potential. The newly developed reference tracer model, on the other hand, compares the kinetics of two *tracers* in the *same tissue* volume, and therefore should be a more accurate model system for quantifying receptor binding.

Figure 5 (a) shows a representation of the dual-reporter protocol. Targeted and non-targeted imaging agents, each fluorescing at a different wavelength, are administered simultaneously and distribute through the bloodstream and extravasate into the tissue. A portion of the targeted drug will bind to nearby cellular receptors. The amount of fluorescence signal originating from bound vs. unbound targeted drug in the tissue will depending on the elapsed time from administration and the rates of binding and exrtavasation. Figure 5 (b) is a representation of the compartmental model system with *K* and *k* denoting the rate constants which describe the movement of the reporters between compartments. A system of rate equations can be written to describe each transport process and combined to produce the equation:

$$C_T(t) = R_1 C_{NT}(t) + \left[k_2 + \frac{R_1 k_2}{1 + BP} \right] C_{NT}(t) * e^{-\frac{k_2}{1+BP} t} \qquad (1)$$

where $R_1 = K_1/K_1'$, $C_T(t)$ and $C_{NT}(t)$ are the time-dependent concentrations of the targeted and non-targeted reporters, respectively, and BP is the binding potential. By measuring $C_T(t)$ and $C_{NT}(t)$ over time, Eq. 1 can be used to fit for the constants, including the binding potential, BP.

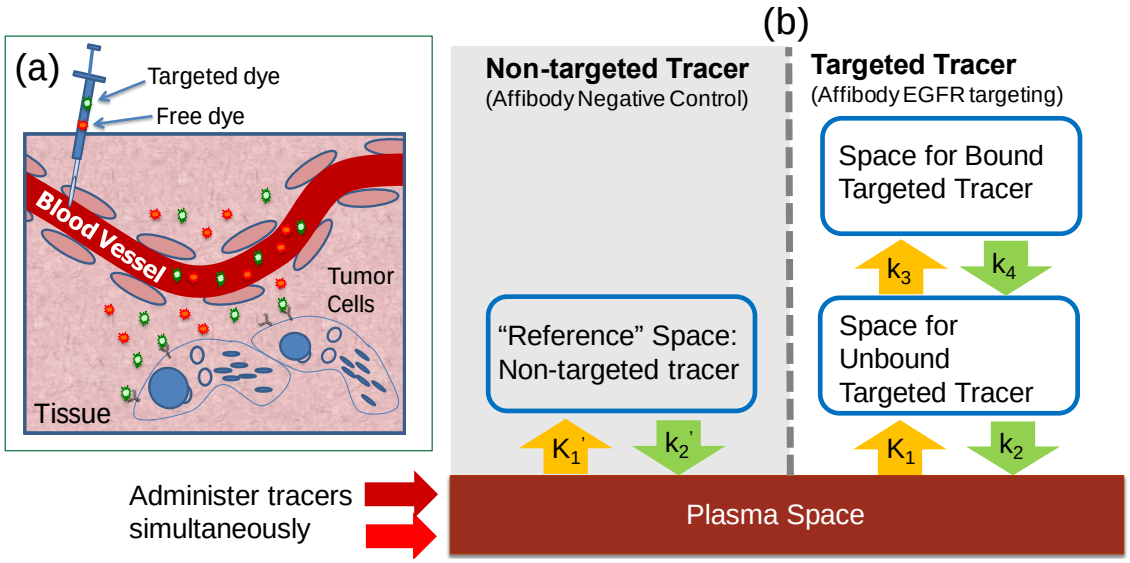


Figure 5. A conceptual representation of the dual-tracer protocol in tissue is shown in (a), which illustrates how targeted and non-targeted fluorescent tracers distribute through the tissue. This process can be approximated with a compartmental model adapted from PET imaging [1] and depicted in (b). Measuring the concentration of the tracers over time allows the model to be fit to the time course data to recover the binding potential (BP), which is a product of the receptor density and the target-receptor affinity.

We have completed a preclinical study to demonstrate quantification of BP of a receptor-targeted imaging agent in orthotoic tumors by simultaneous MR-guided fluorescence molecular tomography (FMT) of

two fluorescent probes in vivo. Nude mice (N=5) were inoculated in the cranium with tumors cells derived from a human glioma line previously shown to over-express epidermal growth factor receptor (EGFR). We chose this model because we have completed extensive characterization of the tumor line and the deeper implantation of the tumors in the cranium provides a more challenging and realistic imaging problem than subcutaneous tumors used for breast cancer models. Immediately before imaging, two NIR fluorescence probes were co-administered in the tail vein (Licro 800CW conjugated to anti-EGFR Affibody Molecule, which targets EGFR, and AlexaFluor 690 conjugated to Affibody Imaging Agent, Negative Control, designed not to bind HER2 or EGFR). After dye administration, mice were imaged for an hour at approximately two minutes per frame using an MR-coupled FMT system.

The imaging system is a spectrometer-based FMT system coupled to a 3T clinical MRI and has been described extensively in previous publications (*2-3*). For this study, the system was modified to acquire data continuously over a specified time interval and data processing routines were updated to process two fluorophores imaged simultaneously. An example of the processing routine for a single source-detector pair is shown in Fig. 6, with the source-detector arrangement shown in Fig. 6 (a). The measured spectrum is shown in Fig. 6 (b) and is made up of fluorescence signals from the targeted and non-targeted reporters, and the tissue autofluorescence. A spectral unmixing technique was used to extract the contributions of each signal to the measured spectrum, as shown in Fig. 6 (c) and (d). The resulting fitted spectrum is represented by the dotted line in Fig 6 (b), which shows an excellent fit to the data. This procedure, which is repeated for each source-detector measurement, allows the signals from the two reporters to be processed separately and removes the autofluorescence signal contamination.

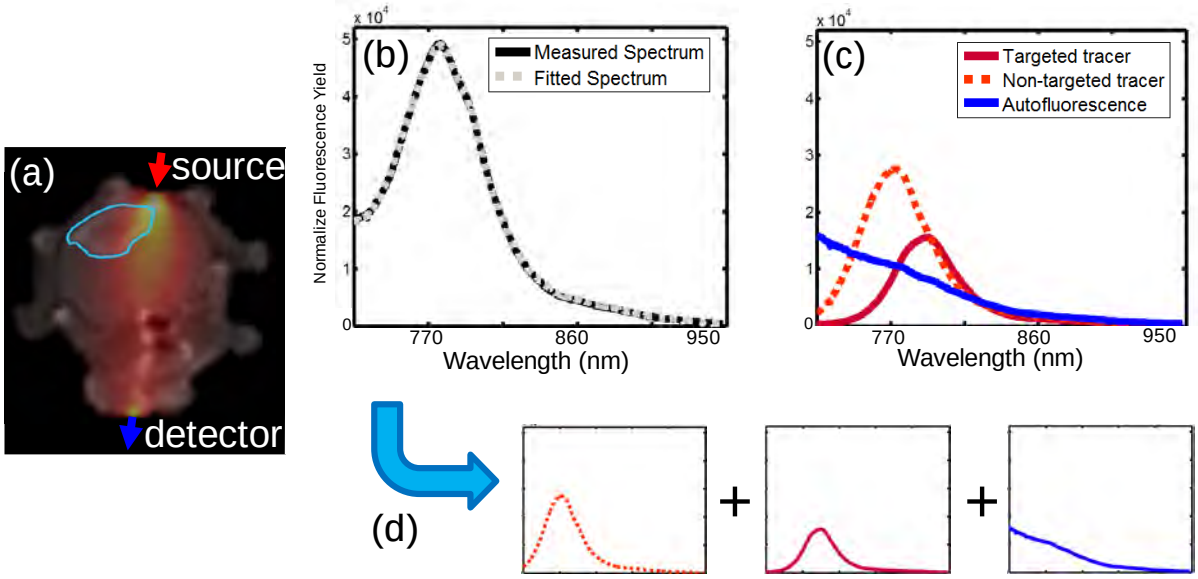


Figure 6. A sensitivity profile of optical signals propagating through tissue is illustrated overlaid on a corresponding coronal MR image of the mouse brain is shown in (a), where the blue line outlines the tumor. A spectrum measured at a detector (b, solid line) is a sum of the fluorescence signals from the two tracers and tissue autofluorescence. Pre-recorded shapes of these spectra, (c), were used to extract the contribution of each tracer and eliminate the autofluorescence contamination (d). The fitted and measured spectra are shown in (b).

The unmixed signals were integrated and used to recovery images of fluorescence yield of both optical probes using MR-guided FMT reconstruction algorithms. A flow chart describing how the data from the optical and MR imaging systems was combined to produce one frame of the imaging sequence is shown

in Fig. 7.

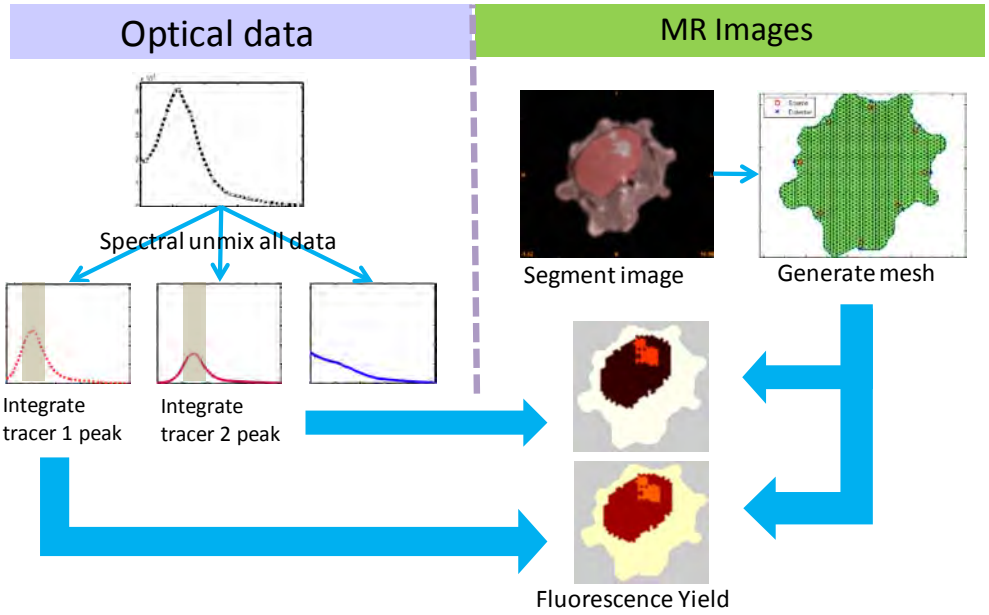


Figure 7. This schematic illustrates how the MRI and optical data were combined to produce images of fluorescence yield for both tracers. Optical spectra were unmixed to produce data for both targeted and non-targeted tracers and the MR images were segmented into general tissue regions. These segmented images and the optical data were combined to produce an image of fluorescence activity in the tissue volume. This process was repeated for every frame in the time series.

Selected images from the full hour-long imaging sequence for of a single animal are show in Fig. 8 (a) for both reporters. These are cross-sectional images of the mouse head with the brain and tumor showing up as internal structures. From these images, the time-course of the fluorescence yield for both reporters in the tumor was extracted and plotted in Fig. 8 (b). It is clear that the kinetic behavior of the targeted and non-targeted reporters is significantly different. The time curves for each animal were used to fit for BP using in Eq. 1 which resulted in a mean binding potential of 0.86 (0.24 st. dev., N=5). Ex-vivo analysis of the receptor density in this tumor line suggests that this figure is reasonable. Full validation of these results is ongoing.

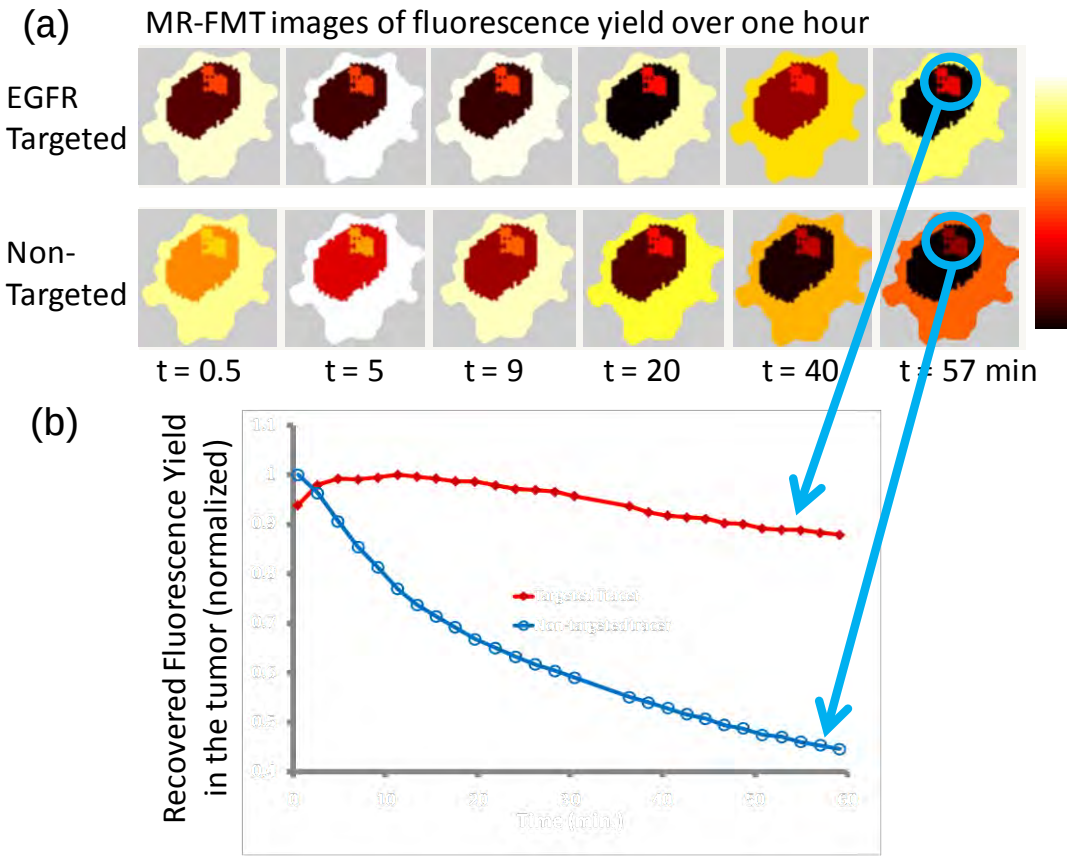


Figure 8. Selected fluorescence yield images of a single coronal slice through the mouse head images for both targeted and non-targeted probes over time are shown for a single animal in (a). The entire time course in the tumor for both tracers is shown in (b). Binding potential values were calculated from the time-courses of the fluorescent probes in the tumor using Eq. 1.

An important implication for this approach is that it accounts for the presence of unbound targeted fluorescent tracer in both the tumor and surrounding normal tissue, much of which is dictated simply by the extravasation and vascular density of the reporter, not the specific binding. We have observed that fluorescence images of exposed tumors may show no or even negative tumor-to-normal tissue contrast for a targeted tracer, while the binding potential maps produce contrasts up to 50 : 1 for the same image series. Thus, this approach may be an important method to enhance contrast for targeted fluorescence guided surgery. I am currently in the process of reproducing these experiments in a planar-imaging geometry suitable for surgical guidance using a HER2 positive tumor line. By exchanging the anti-EGFR Affibody Molecule with the anti-HER2 Affibody Molecule on the Licor800CW, the same fluorescence reporters will be used. In addition to broad-beam surgical imaging systems, the spectrometer-based probe system described above is an ideal measurement system for the dual-reporter approach.

Key research accomplishments

- Developed and characterized a new spectrometer-based probe system for quantifying fluorescence activity in excised tissue specimens.
- Demonstrated the ability to measure endogenous PPIX fluorescence in excised breast tumor models (MTGB tumor line).

- Contributed to the development of a dual-reporter compartmental model to quantify the binding potential of a targeted fluorescence reporter in tissue.
- Demonstrated quantification of binding potential of EGFR in orthotopic tumor models using MRI-coupled FMT.

Reportable outcomes

During the second year of this grant, both papers submitted during the first year (reported in last years' report) were accepted and published. The second year of this traineeship grant has two first author manuscripts in preparation and one second author paper in preparation. In addition to attending seminars and conferences, I also served on the organizing committee and as a session chair for the Engineering in Medicine Symposium: Redesigning Cancer Imaging and Therapy, at the Thayer School of Engineering, Dartmouth College (July, 2011).

Conclusions

Progress has been made on developing tools to identify tumor margins and in excised tissue specimens and in vivo. Extensive studies with a newly developed fluorescence probe and the associated spectral unmixing algorithms demonstrated highly linear responses to fluorophore concentration, though changes in the tissue optical properties had an unacceptably high impact on the measurements. A second generation probe system, which should further mitigate the effects of tissue optical properties, is nearing completion and will be tested extensively in phantoms and tissue specimens. Additionally, a new approach to quantify receptor binding potential, a critical parameter in targeted fluorescence imaging, has been developed and tested in animal models. This approach has the potential to dramatically improve tumor-to-normal contrast by mitigating the effects of drug kinetics and emphasizing the reporter contrast due only to binding. Thus, it has enormous potential for drastically improving the identification of tumor remnants in surgical cavities using either fluorescence probe or broad-beam imaging systems.

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